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14. ABSTRACT

Telomerase, a ribonucleoprotein enzyme composed of an RNA template (hTR) and a catalytically active protein subunit (hTERT), synthesizes telomeres after cell divisions and is obligatory for continuous tumor cell proliferation as well as malignant progression of breast cancer cells. Telomerase is an attractive anti-cancer therapeutic agent because telomerase activity is present in over 90% of human breast cancers but is undetectable in most normal somatic cells. Traditional therapies (surgery, chemotherapy, radiotherapy, etc.) lack the ability to effectively control and cure breast cancer, primarily because residual cells are or become resistant to DNA damaging modalities including standard chemo- and radio-therapies. Since telomerase requires its associated hTR for repeat synthesis, we have chosen to use RNA interference as a method to inactivate hTR and hence telomerase. RNA interference (RNAi) has become a powerful tool for the analysis of gene function in that RNAi allows sequence specific inhibition of gene expression. Another protein we targeted is p21, which has long been established as a requirement for senescence. We wanted to further examine its relationship to senescence and apotosis, in an attempt to sensitize breast tumor cells more effectively.

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Table of Contents

Introduction

Last year over 211,000 new cases of invasive breast cancer occurred among women in the United States (American Cancer Society, 2005). Currently, the primary treatment for breast cancer consists of surgery and adjuvant therapies including chemotherapy, hormone therapy, and localized radiation. Despite the initial success of these clinical approaches, the frequent recurrence of breast cancer indicates that resistance to therapy is common in breast tumors. Associated with nearly 90% of malignant breast cancer, telomerase is a reverse transcriptase containing a catalytic protein component, hTERT, and an RNA template, hTR, for catalyzing the addition of telomeric (TTAGGG) DNA repeats onto the chromosome ends (8,18). As such a prominent molecular marker for human cancer, telomerase has proven useful for detection of recurrent disease, as well as a promising target for adjuvant cancer therapy, especially for breast cancer treatment (15). Traditional therapies (surgery, chemotherapy, radiotherapy, etc.) lack the ability to effectively control and cure breast cancer, primarily because residual cells are or become resistant to DNA damaging modalities including standard chemo- and radiotherapies. Since telomerase requires its associated hTR for repeat synthesis, we have chosen to use RNA interference as a method to inactivate hTR and hence telomerase. The siRNAs we will use are directed at the hTR portion of telomerase, which is a modification of the traditional RNAi in that hTR is a functional RNA and not an mRNA RNA interference (RNAi) has become a powerful tool for the analysis of gene function in that RNAi allows sequence specific inhibition of gene expression (1,4,9-14).

Furthermore, we have elected to examine the effects of inhibiting telomerase by blocking hTERT, using a dominant negative (DN) and/or siRNA, and determining the differences in induction of breast cancer cell sensitivity to standard breast cancer therapies. We have found decreased telomerase activity using both hTR siRNA and DN-hTERT in MCF7 breast tumor cells. Preliminary experiments have shown decreased telomerase activity using the telomere repeat amplification protocol (TRAP), in certain clones of MCF7 cells containing hTR siRNA as well as DN-hTERT cell lines as compared with controls. Over time we have observed telomere shortening, senescence and an increase in sensitivity to Adriamycin (AdR) at lower levels than standard treatments in our DN-hTERT clones, suggesting that the telomeres are more vulnerable to AdR. The increased susceptibility of the cells to DNA damage will be critically important in the induction of apoptosis or senescence, and blocking telomerase will likely prevent proliferative recovery in both sets of cell lines. The experiments here should provide new perspectives on RNAi and clarifications of the cellular response of breast tumor cells to treatment after sensitization by telomerase inhibition, which will be critically important for the identification of adjuvant therapies directed at telomerase for breast cancer patients. Another protein we targeted is the cyclin-dependent kinase inhibitor, $p21^{waf-1}$, which has long been established as a requirement for the onset of cellular senescence. We wanted to further examine its relationship to senescence and apoptosis after treatment of breast tumor cells, in an attempt to sensitize these cells more effectively in hopes to reduce collateral effects on surrounding normal cells.

Body

To date, we have utilized the established MCF-7 breast cancer to study the effects of the targeted siRNAs. Initially, we created two siRNAs that target different portions of the functional hTR RNA. The first position is located in the single stranded template region, which will be called hTR-T, and the other is complimentary to the conserved pseudoknot domain (CR2/CR3) domain, which will be called hTR-2 (Figure 1 & Table 1) (3).

As a first step, synthetic siRNAs were created that are 21 nucleotides in length paired in a manner to have 2 nucleotide 3' overhangs on each end. Each siRNA was transiently transfected into MCF-7 cells in two separate experiments. We observed an approximately 80% reduction in telomerase activity over a 72-hour time period in the hTR-2 treated cells and a slightly smaller reduction in the hTR-T treated cells (Figure 2 & data not shown). This provides the necessary proof of

principle experiment demonstrating that the vector-mediated expression of these siRNAs will inhibit telomerase. In addition, the preliminary experiments are noteworthy in that RNAi is normally regarded as limited to the cytoplasm, but because telomerase is predominantly nuclear, it is quite possible that the knockdown of telomerase transpired in the nucleus.

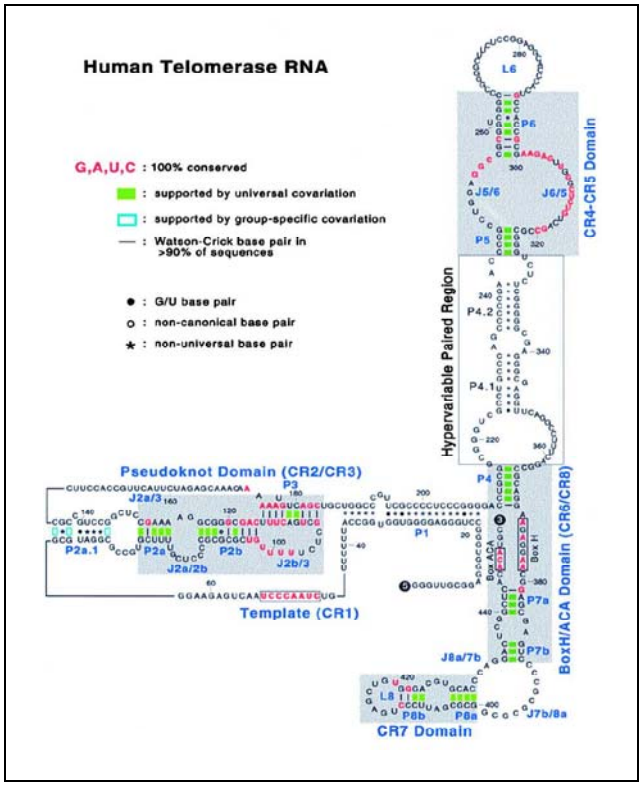

Figure 1. Proposed Secondary Structure of Human Telomerase RNA. This schematic displays the proposed L-shape secondary structure of the functional hTR RNA. There are 10 conserved helical regions (P2a-P8b), which constitute the four universal structural domains known as the pseudoknot domain, the CR4-CR5 domain, the Box H/ACA and the CR7 domain. These are all shaded in gray and labeled. Note the single-stranded and labeled template region, which we targeted with hTR-T (taken from Chen et al. 2000 (3)).

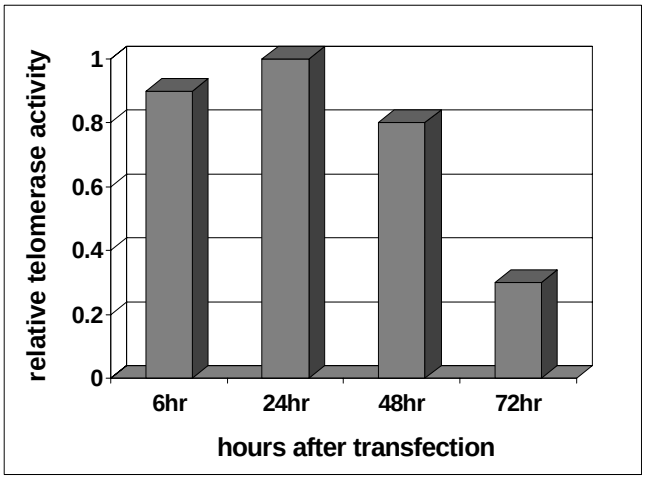

Figure 2. Decline in telomerase activity using siRNA targeted against hTR. siRNA was designed and directed against a critical pseudoknot (hTR-2) found in the predicted secondary structure of the telomerase RNA template, hTR. After transfection, cells were harvested and tested for telomerase activity using the telomere repeat amplification protocol (TRAP) at the indicated times. The relative telomerase activity was calculated as a percentage of the 24-hour time point. We observe a reproducible 3 to 4-fold decline in activity after 3 days of treatment.

In order to determine if telomerase could be knocked down further and for a greater length of time, we chose to stably express these sequences into the MCF-7 cells. The pSUPER-retro vector, which is specifically designed for RNAi, enabled us to accomplish this task using retroviral infection and selection with puromycin for stable integration of the vector sequences. After transcription/expression of the siRNA, the RNA sequence will fold into a 64 base pair hairpin loop that will be cut *in vivo* by a

Ribonuclease III enzyme called dicer, creating the same length 21bp siRNAs as the synthetic version (2). Thus far, the hTR-T and hTR-2 sequences have been cloned into the vector and have been successfully infected into MCF-7 cells followed by cylinder cloning.

Objective #1: **Maintain stable suppression of hTR using RNA interference with high levels of telomerase inhibition.**

MCF7 cell lines infected with hTR-T and hTR-2 were screened using the TRAP assay to find the clones with the greatest decrease in telomerase activity. Unfortunately, most clones did not display large decreases in activity, averaging approximately 50% knockdown (Figure 3) (hTR-2 data not shown). Perhaps the retroviral efficiency was not as high as expected so we decided to shift to a lentiviral system due to the ability to infect dividing as well as non-dividing cells, therefore increasing the infection efficiency.

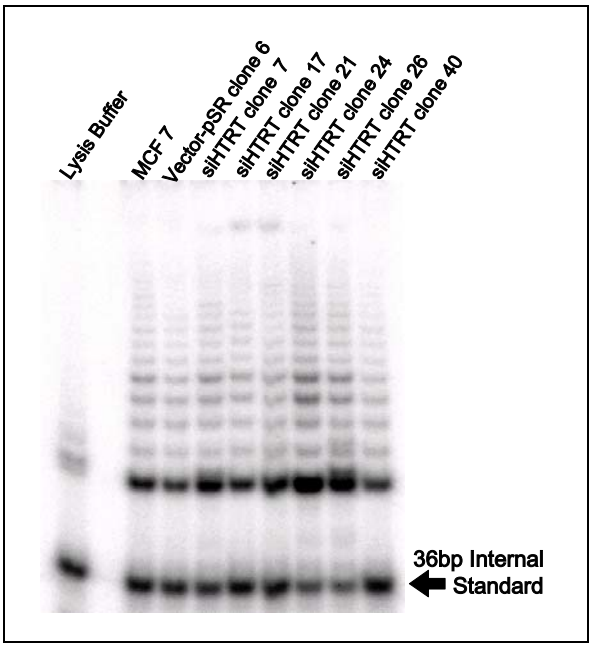

Figure 3. Decreased telomerase activity is found in certain clones of MCF7 as compared with controls. The MCF7 breast cancer cells were stably infected with siRNA directed at the template region of the RNA component of telomerase, hTR. Activity was only decreased in a few of the clones (lanes 5,6,9) after siRNA infection as assessed by TRAP. Knockdown of telomerase was ~ 50%.

We obtained the lentivectors from Dr. Elizabeth Blackburn's laboratory, which express siRNAs targeting hTR that completely knockout telomerase activity in a variety of cell lines (11). Thus far, the vectors have been infected into the MCF7 cell lines and are currently undergoing selection. We will utilize these lines to test for sensitization of tumor cells to common breast cancer therapeutic agents

Alternative 1: We will utilize a siRNA system developed by Dr. Elizabeth Blackburn's laboratory, where they express hTR siRNA in a lentivral system while co-expressing either exogenous wild-type hTR or 2 template mutant versions (11). However, it is quite possible that if this siRNA for hTR is potent, no stable populations will be obtainable.

***Objective #2*: Determine if siRNA knockdown of telomerase sensitizes breast tumor cells to conventional breast cancer therapy.**

Here, we will examine the relationship between telomere length and the DNA damage caused by cancer treatments including Adriamycin (AdR), γ-radiation, and etoposide. In this portion of the study, we are going to attack the cells in two ways: first, by blocking telomerase with the siRNAs, and second, by the addition of one of the three cancer treatments. The first set of cells we will be using

will have functional p53 (MCF-7 & ZR-75) and the second set will lack p53 expression (T-47D & MDA-MB231). Wild-type p53 plays an important role in the cellular response to cell damage in that when p53+ cells are treated with AdR, cells undergo replicative senescence; however, cells without p53 undergo a delayed apoptosis after treatment with AdR (5,7).

Assess the effect of the siRNAs individually as a sensitization pretreatment to different types of breast cancer therapy: Our strategy is to suppress telomerase activity using the siRNAs in an attempt to sensitize the breast tumor cells to therapeutic agents, thus causing a more effective cellular response at a less toxic dose. The four cell lines will be infected by the lentivectors with and without the siRNAs, selected with puromycin (0.6μg/μl) and grown to confluency. Then the cells will be split appropriately and exposed for 2 hours to the following therapeutic modalities: AdR (0.1 to 3 μM), etoposide (1-100 μM), and γ-radiation (0.1-10 GY). For all cell lines, there will be at least three replicate plates for each: one for AdR, one for etoposide, and one for γ-radiation. This entire study will be replicated exactly for the each hTR siRNA construct. We will test the breast tumor cells for apoptosis (TUNEL), telomere lengths (TRF & FISH with a telomere specific probe), telomerase activity (TRAP), senescence (SA-βgal), and cytogenetic alterations such as end fusions, telomere breakage or ring chromosomes (karyotyping & SKY).

Alternative 1: Our laboratory has found that treatment of MCF-7 (p53+) cells induces widespread senescence within the population (more than 99.99%) (5). However, recovery after a single acute dose of AdR consistently occurs and these cells always express telomerase activity (7). Thus, we will look at proliferative recovery in p53 and breast tumor cells to determine if inhibition of telomerase is capable of preventing outgrowth of resistant or recovered cells. This will be done quantitatively by determining the number of recovered clones with and without hTR siRNA at the dose range indicated.

Alternative 2: One possibly important issue in knocking down hTR as a therapeutic target would be its expression in normal cells, as it is ubiquitously expressed. This alternative would involved the use of normal or preimmortal mammary epithelial cells (6), which express hTR but not hTERT, as a means to determine the sensitization of these cells to similar therapeutic modalities as above.

Expected Results: The decreases in telomerase levels and telomere length caused by the pre-treatment with the hTR siRNAs will ultimately make the telomeres more vulnerable to the AdR, etoposide and γ-radiation. This will result in higher levels of telomere dysfunction and chromosomal abnormalities due to the lack of telomeric repeats to protect the chromosome ends. The increased susceptibility of the cells to DNA damage will be critically important in the induction of apoptosis

***Objective #3*: Determine if knockdown of telomerase using DN-hTERT sensitizes breast tumor cells to conventional breast cancer therapy.**

The rate-limiting component of the telomerase complex is hTERT expression levels. Somatic cells contain only hTR and are without telomerase activity, whereas the majority of tumorigenic cells have telomerase activity because they express hTERT. Therefore, we want to look at the effects of telomerase inhibition using DN-htert and consequently blocking telomere maintenance in breast tumor cells.

Evaluate the effects of DN-hTERT in MCF7 cells: We obtained the catalytically inactive, dominant negative form of hTERT from Hahn et al., which has two substitutions in the third RT motif of hTERT. At positions 710 and 711 the aspartic acid and valine residues were switched to alanine and isoleucine

respectively. The retroviral vector was introduced into MCF7 cells followed by selection with puromycin for several days. Telomerase activity was analyzed in clonal isolates and reduced activity was found in all of the populations with the greatest knockdown in clones 2, 6, and 10 (Figure 4). The level of expression of the DN-hTERT vector for each cell line was also ascertained using RT-PCR (Figure 5). Clonal variability seen in the differences of decreased telomerase activity correlated with the amount of DN-hTERT RNA quantitatively, so those clones with greater expression of DN-hTERT had lower levels of telomerase activity.

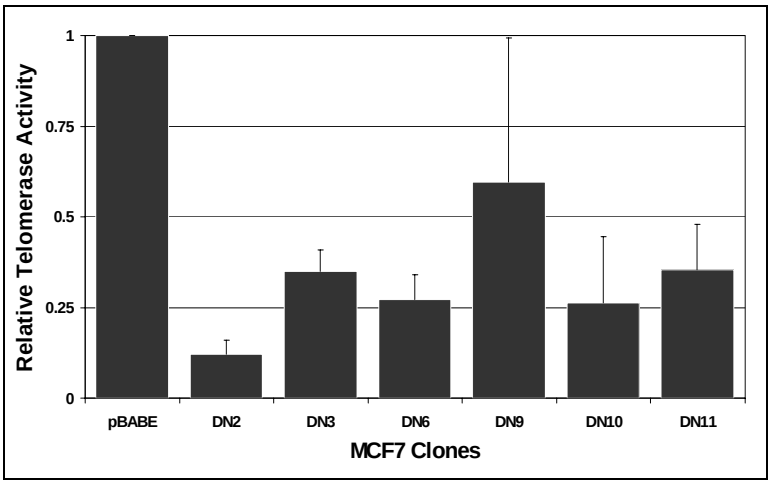

Figure 4. DN-hTERT causes decreases in telomerase activity in clones of MCF7 cell lines as compared to controls.
The MCF7 breast cancer cells were stably infected with dominant negative hTERT. Telomerase activity was determined using the TRAP assay. In several of the clonal populations (2,3,6,10) knockdown of telomerase was 80% or higher.

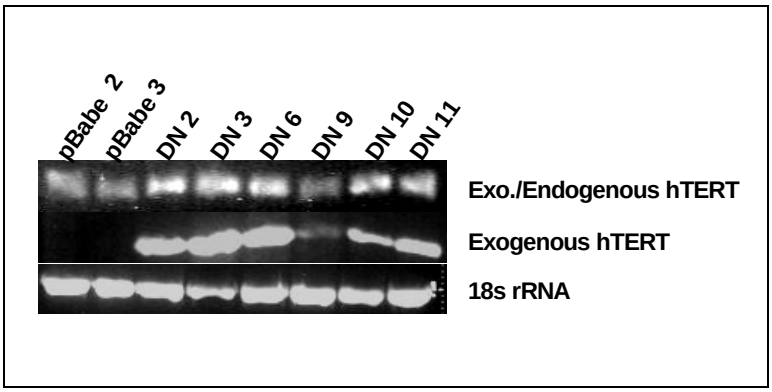

Figure 5. Expression levels of hTERT were assessed.
RT-PCR was conducted to determine the expression of both the constitutive levels of hTERT as well as the introduced DN-hTERT levels**.**

Next, I looked at telomere lengths in order to discover if inhibition of telomerase via DN-hTERT resulted in telomere shortening. We found an ongoing rate of loss as time progressed. As compared to normal MCF7 and pBABE control cell lines, the different clones displayed a marked decrease in telomere length of 1Kb or more (Figure 6). The amount of shortening appears to correlate with level of DN-hTERT expression.

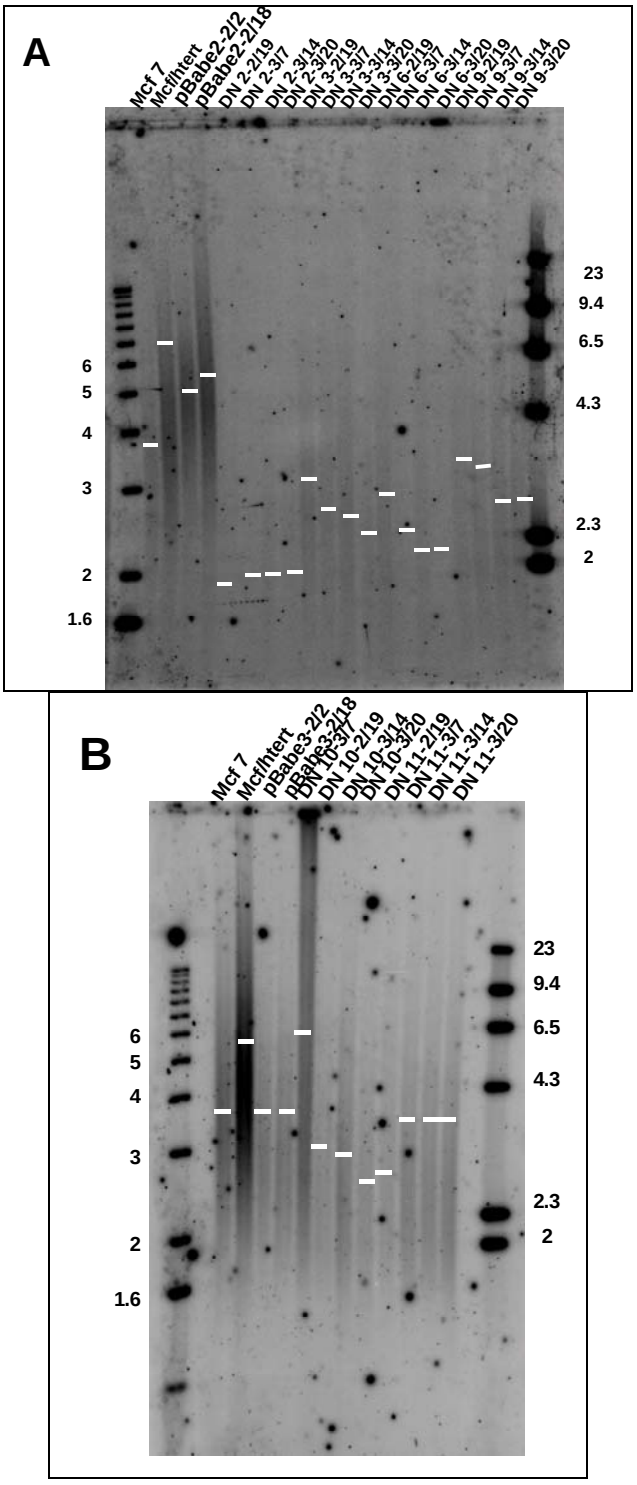

Figure 6. DN-hTERT causes gradual telomere shortening.
Telomere lengths determined by the TALA assay showed marked shortening in specific clones of DN-hTERT/MCF7 cell lines (2,3,6,10). Samples were sequentially collected over a month's time for six different clonal populations. White bars indicate median telomere length as calculated using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Approximately, one-month post-infection cell growth slowed and morphological changes occurred within different populations. The cells became large and flattened in appearance as well as the incidence of cell death. We stained the various clones for senescence using β-galactasidose (β-gal) staining and established the extent of apoptosis through the TUNEL assay (Figure 7). Some apoptosis was found but at this stage the majority of the cells were undergoing senescence.

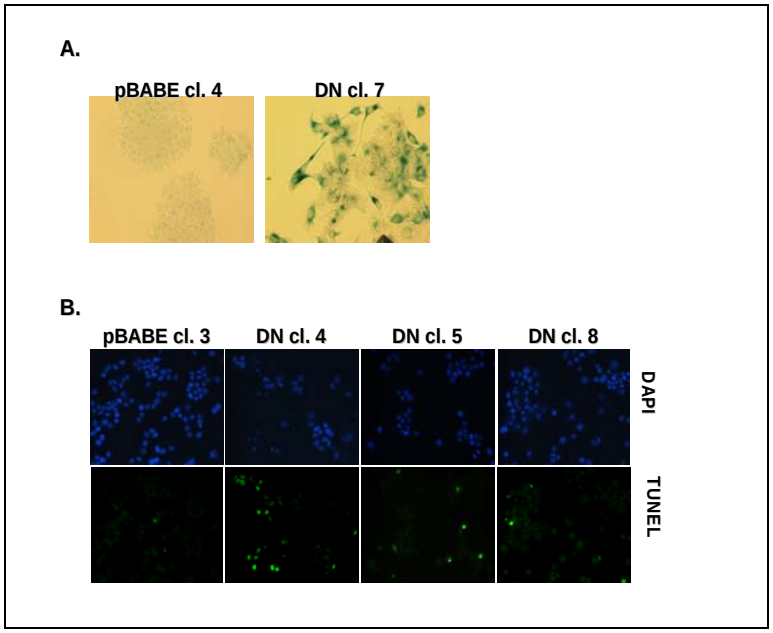

Figure 7. Senescence and increased apoptosis is observed in DN-hTERT/MCF7 clones. Some MCF7 clones displayed a senescent phenotype and high levels of staining for β-gal about one month post-infection with DN hTERT (A). Using the TUNEL assay, elevated levels of apoptosis were detected in the dominant negative hTERT/MCF7 clones, although most cells undergo senescence (B).

Unexpectedly, after this initial response of crisis, defined by growth arrest, critical telomere shortening, senescence and apoptosis, the clonal populations recovered. From these long-term cultures emerged surviving cells that reverted back to normal phenotype and growth rate (data not shown). Furthermore, the cells regained the high levels of telomerase activity as found in normal MCF7 and pBABE control cell lines (Figure 8).

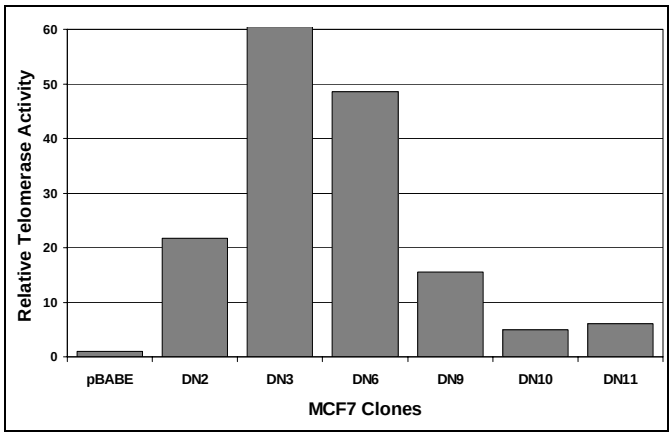

Figure 8. Loss of telomerase repression and recovery. Approximately 4 months post-infection, the DN-hTERT cells went through "crisis" and telomerase activity was detected as a measure of recovery.

Reasons for this recovery are as of yet unknown. However, two possibilities exist: the first being inactivation of the DN-hTERT transgene and the second increase in endogenous hTERT transcription with the persistence of the DN transgene expression.

Assess if pre-treatment with DN-hTERT causes increased sensitization to traditional breast cancer therapy: Three of DN-hTERT clones with different amounts of telomerase activity were selected approximately 3 months post-infection for sensitization by AdR. Approximately, 2000 cells were seeded, and the next day we administered an acute AdR treatment for 2 hours with varying concentrations (0.0-0.75 uM). Ten days later plates were stained and colonies counted. In comparison

to the MCF7 and pBABE cell lines, all of the DN-hTERT clones exhibited a greater sensitivity to AdR (Figure 9). Clonal growth rates were ascertained as critically lower than controls in almost all of the concentrations.

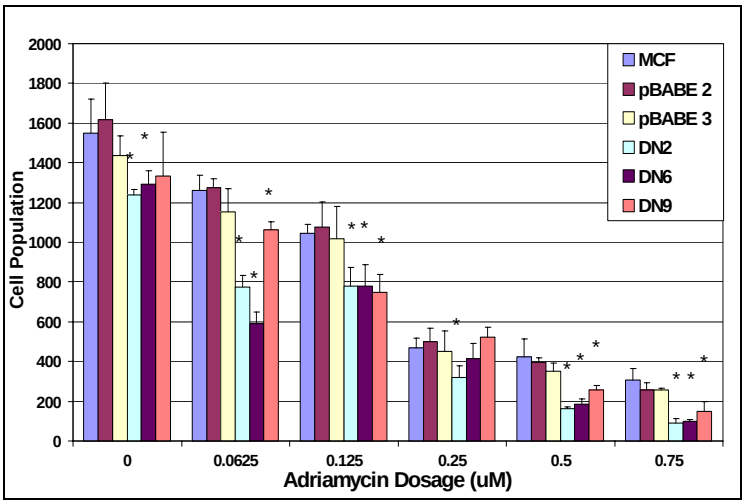

Figure 9. DN-hTERT expression confers ncreased sensitivity after AdR treatment.
DN-hTERT clones displayed significant increases sensitivity as seen by decreased growth after treatment as well as at lower dosages. Clonal differences in sensitivity correlated to levels of DN-hTERT expression within the population. Note: The * indicates a significant p-value >0.05 as calculated in a paired t-test.

This finding implies that increase susceptibility of the telomeres to AdR and thereby DNA damage, which will be significant in the induction of apoptosis or senescence. Perhaps we may be able to prevent proliferative recovery by blocking telomerase. These observations demonstrate that the disruption of telomere maintenance limits cellular lifespan in human cancer cells thus validating hTERT as an important target for the development of anti-neoplastic therapies.

Alternative 1: I will repeat the same sensitization study using different chemotherapeutic agents, etoposide and taxol. Also a topoisomerase II inhibitor, etoposide should provide similar sensitization results. However, taxol is not a telomere specific drug and does not cause apoptosis through telomere dysfunction. Both will provide a more complete analysis of the sensitization caused by DN-hTERT.
Alternative 2: Instead of using DN-hTERT, I will create two HTERT siRNAs and infect MCF7 cells followed by the same characterization as seen above with the MCF7/DN-hTERT cell lines. This will provide another means of inhibition as well as a comparison in the breast cancer cell sensitization studies.

Objective # 4: **Determine if siRNA knockdown of p21 sensitizes breast tumor cells to convential breast cancer therapy.**
Our lab has previously shown the molecular and cellular consequences of Adriamycin treatment in breast tumor cells. After acute exposure to Adriamycin, MCF-7 cells senescence approximately three days later and down-regulate telomerase. Telomere length has been proven to be an important trigger for senescence. However, we have shown that senescence can be induced without net shortening of the telomeres. That the most critical event is the preservation of telomere structure/integrity and p53 levels only when this occurs do we have AdR-induced senescence in MCF-7 cells. Adriamycin and ROS have been shown to preferentially attack the telomeres. Senescence is also characterized by transient p53 activation, high levels of reactive oxygen species as well as sustained p21 expression. From these results I want to further examine and understand the relationship between p21 and senescence. Perhaps there is a threshold of p21 needed to maintain the senescence phenotype, and if I

can knock down the p21 protein levels enough then the cell should undergo apoptosis instead of senescence.

Determine if p21 can be sufficiently inhibited in breast tumor cells using RNAi: We have already established two cell lines that have significant p21 knock down with siRNA targeting exon 3 (325-343), MCF/p21-4 clone 3b and MCF/p21-4 clone 11b. As stated previously, the inhibition of p21 was not sufficient enough to be maintained post-AdR treatment. So the next step was to obtain another p21 siRNA sequence, which targets farther downstream, also in exon 3 (400-420), and conduct an infection. The new sequence was introduced into the old cell lines, and the pSUPER-retro vector was again utilized for the second infection, except this one has a puromycin selectable marker. Once infected into the cell lines and selected, the clones were screened for constituitive levels of p21 by Western blotting. There were varying levels of knockdown with the greatest ranging around 100% which is greater than the original knockdown seen in the first p21 cell lines created. Then cell lines containing both siRNA constructs were exposed to an acute treatment with AdR (2 hours). Levels of p21 increased immediately with detectable levels seen at zero hours after dosage and peaking at fours hours post-treatment (Figure 10).

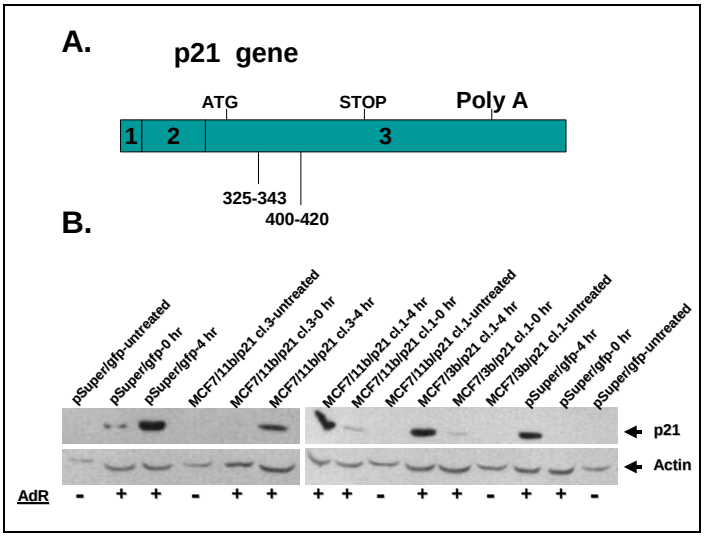

Figure 10. Down-regulation of p21 using siRNA and the relative levels of p21 induction after AdR treatment. Previously established cell lines MCF7/3b and MCF7/11b with p21 knockdown were infected with another siRNA targeting p21 (A). Constituitive levels of p21 were decreased over 90% in most of the clones. Elevated levels of p21 were detected post-AdR treatment (0 & 4 hours) even with the presence of two siRNAs per cell line (B**).**

We have clearly shown that blocking p21 function results in a transient decrease after treatment with Adriamycin followed by a large amplification in protein quantities. This constitutive 80% knockdown of p21 levels does not appear to be significant enough to be maintained post treatment with AdR. We observed the majority of the cells senescencing while others appear to undergo apoptosis. The reason for this result is still unknown and is under investigation. Because we see p21 knockdown with siRNA before but not after DNA damage, we will explore the option of homologously knocking out p21 in the MCF-7 cell line as a cleaner system for p21 knockout.

Key Research Accomplishments

1-Athough transient inhibition of telomerase activity was observed; using RNAi to knockdown levels of hTR was not as effective as expected using the retroviral pSUPER system. A significant stable decrease in telomerase activity was not observed in culture.

2-Even though not complete, significant knockdown of telomerase activity using DN-hTERT resulting in decreased telomerase activity, telomere shortening, senescence and apoptosis.

3-DN-hTERT caused increased susceptibility of the telomeres to AdR resulting in significant differences in colony growth after acute treatment

4-The unexpected finding of recovery of telomerase in the DN-hTERT cell lines after a certain amount of time indicating a window of opportunity for treatment and suggests that telomerase is an ideal marker for tumor cell recovery.

5-Levels of p21 were decreased 50-90% using RNAi and multiple siRNA targets in breast tumor cell lines.

6-Acute AdR treatment of MCF-7/p21 knockdown cell lines causes an induction of p21, and the RNAi occurring in these cell lines does not suppress DNA damage-induced activation of p21 after treatment.

Recommended Changes to the Proposed Work Based on Additional Findings

In order to complete the studies in *Objective* #1 related to *in vitro* and *in vivo* study of RNA interference, I will be utilizing the lentiviral system developed in Dr. Elizabeth Blackburn's laboratory (11) for the rest of the hTR studies in breast tumor cells, as well as test for sensitization with the dual expression of hTR siRNA and mutated hTR sequences. I will also be testing synthetic siRNA pools for inhibition telomerase activity by blocking hTR and attempt to define the mechanism of telomerase inhibition. In addition, I will be looking at the mechanism of recovery in the MCF7/DN-hTERT cell lines as well as a comparison of levels of induced sensitivity of breast cancer cells after telomerase inhibition. As was the case last year, the addition of the p21 knockdown characterization will continue.

Reportable Outcomes

Abstracts/Presentations

Poynter, K.R., Elmore, L.W., Holt, S.E. VCU Institute for Women's Health: 2nd Annual Women's Health Research Day. Richmond, VA. March 2006.

Poynter, K.R., Elmore, L.W., Holt, S.E. VCU: Massey Cancer Center Retreat. Richmond, VA. June 2006.

Poynter,K.R., Elmore, L.W., Holt, S.E. VCU: Daniel T. Watts Research Poster Symposium. Richmond, VA. October 2006.

Manuscripts

Poynter, K.R., Elmore, L.W., Holt, S.E. 2006. Telomeres and telomerase in aging and cancer: Lessons learned from experimental model systems, Drug Discovery Today: Disease Models. **3**:155-160.

Development of Cell Lines

We have developed cell lines for telomerase knockdown with a lentiviral system targeting the template region of hTR and using a dominant-negative approach to blocking telomerase (DN-hTERT). In addition we created two cell lines with a double knockdown via RNAi of p21 in MCF-7 cells. Other siRNA vectors that have been constructed and expressed in breast tumor cells but not completely characterized include several MCF-7 cell lines that have targeted knockdown of hTERT, using RNA interference.

Funding Applied For

Other than the Department of Defense Breast Cancer Research Program, Pre-doctoral award, awarded May 2003, no additional funding has been applied for.

Conclusions

Having explored several avenues of RNA interference, *in vitro* using synthetic siRNAs and *in vivo* using retroviral infection of siRNAs, we are clearly on pace to determine what occurs when directing this mechanism at a functional RNA. Using RNAi to knock-down levels of hTR was not as effective as expected. The significant decreases in telomerase activity that were expected were not observed *in*

vivo. However in our DN-hTERT clones, we have also shown telomere shortening, senescence and an increase in sensitivity to Adriamycin (AdR) at lower levels than standard treatments. Thereby, suggesting that the telomeres are more vulnerable to AdR. In addition, utilizing siRNAs to attack p21 has lead to the discovery that this protein is capable of returning to greater than normal levels after treatment with adriamycin for two hours. The resulting phenotype in these cell lines from AdR treatment remains somewhere in between senescence and apotosis. Inhibition of telomerase and p21 in breast tumor cells in combination or alone may provide a novel mode of cancer therapy and will be critically important for the rationale design of new adjuvant therapies for breast cancer patients.

Abbreviations

hTERT-human telomerase reverse transcriptase; **hTR**-human telomerase template RNA; **AdR**-adriamycin; **siRNA**-short interfering RNA; **RNAi**-RNA interference; **SKY**-spectral karyotyping; **FISH**-fluorescent *in situ* hybridization; **SA-β gal**-senescence associated β -galactosidase; **ChIP**-chromatin immunoprecipitation; **TRAP**- telomere repeat amplification protocol (telomerase activity assay); **TRF**-terminal repeat fragment (telomere length assay); **TRF2**- telomere repeat binding factor number 2; **IP**-immunoprecipitation; **TnT**- transcription and translation.

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Telomeres and telomerase in aging and cancer: Lessons learned from experimental model systems

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Telomerase is expressed in nearly 90% of human tumors, making it a promising therapeutic target and diagnostic aid. Recently, there has been an explosion of new model systems for studying the roles of telomerase and dysfunctional telomeres in aging and cancer. Here, we describe models that are proving particularly relevant for studying the function and regulation of telomerase as well as the biological consequences of its inhibition. A critical comparison of these models is included, highlighting strengths and weaknesses as they relate to cancer and aging in humans.

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Introduction

After heart disease, cancer is the second leading cause of death, and the occurrence of cancer is strongly tied to advanced age [1]. Genomic instability, including telomere dysfunction, is thought to be a major contributory factor in aging and progression to cancer. Telomeres, the natural caps of linear eukaryotic chromosomes, serve to maintain and protect chromosomes from being recognized as double strand breaks, eliciting nonhomologous end joining that results in telomere fusions [2]. Normal somatic cells continually shorten their telomeres with each cell division (~50–100 bp lost) [3]. Progressive telomere shortening correlates with a decline in proliferative capacity and has been linked with induction of the irreversible growth arrested state known as replicative senescence.

Telomerase, a reverse transcriptase that catalyzes the addition of telomeric DNA repeats (TTAGGG) onto the ends of eukaryotic chromosomes, is activated in 85–90% of human cancers [4]. The telomerase enzyme preserves telomere length allowing for indefinite cell proliferation [5]. As such ubiquitous markers of cancer, there is a clear need for biologically relevant model systems to study how telomeres and telomerase contribute to tumorigenesis and aging, a well-established cancer risk factor.

In vitro models

The cloning and then stable expression of hTERT, the catalytic component of human telomerase, into normal cells allowed for direct proof that telomeres are an internal biological clock. This provided the molecular basis for the long-appreciated Hayflick Limit, a phenomenon in which normal cells in culture only have a finite numbers of population doublings (i.e. lifespan). Without a telomere maintenance mechanism, cells undergo replicative senescence [3]. Cells ubiquitously express the RNA component of telomerase (hTR for humans) and the chaperones necessary for proper folding. Therefore, ectopically expressing hTERT allows for the recon-

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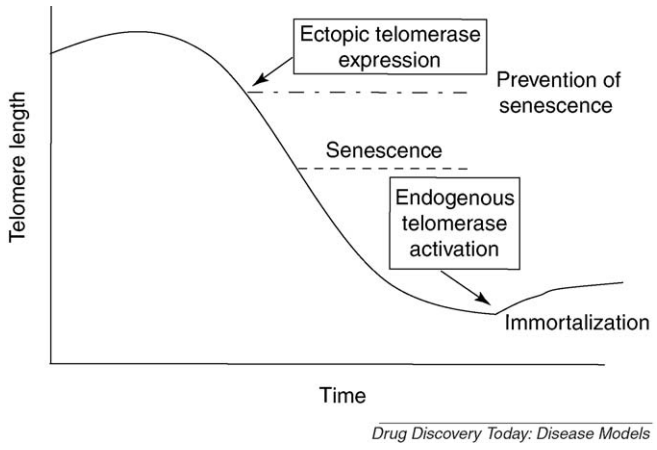

Figure 1. Schematic representation of cellular aging in terms of telomere length. Normal cells will only proliferate for a certain number of population doublings, followed by cellular senescence. Some cells are capable of inactivating tumor suppressor proteins, thereby bypassing senescence and allowing continued cell growth and telomere erosion. Ultimately, cells will enter crisis, presumably owing to crucially shortened or absent telomeres, and those capable of immortalization reactivate telomerase. In addition to normal cell lifespan and telomerase activation, this model displays the ability of ectopic telomerase to prevent senescence and extend the lifespan of cancer prone cells, thereby protecting against their tendency to become genomically unstable and to spontaneously immortalize, eventually leading to transformation.

stitution of enzymatic activity, providing a useful model system to test the effects of stably expressing telomerase in normal cells and cells derived from different disease conditions.

Exogenous telomerase maintains or elongates telomeres in a wide variety of cell types while extending *in vitro* lifespan [6,7]. This apparent indefinite proliferative potential is not accompanied by other hallmarks of malignant cells such as genomic instability, loss of contact inhibition and the ability to form tumors in nude mice [8]. Thus, telomerase-expressing normal cells are clearly distinct from classically immortal cells, with potential in the antiaging and tissue replacement arenas. Introducing hTERT into senescent cells has been unsuccessful at reversing cellular aging, but cell cultures with only a few doubling remaining could be rescued [9]. Although this wide window of efficacy is appealing both practically and technically, the fact that cells accumulate DNA damage throughout their lifespan could have biological implications. As discussed below, one important lesson learned from *in vitro* model systems has been that the biological impact of telomerase expression is highly context-dependent.

Besides allowing normal somatic cells to bypass replicative senescence, ectopic telomerase is also able to extend the lifespan of cancer prone cells while protecting against their tendency to become genomically unstable and to classically immortalize [10]. This has led us to propose that telomerase might represent a unique, perhaps multifaceted, tumor suppressor (Fig. 1). Telomerase does not become functionally inactive and then contributes to the development of tumors according to the classic tumor suppressor paradigm. Instead, if expressed before genomic instability, ectopic telomerase can have anticancer and antiaging effects by helping to suppress the instability. However, in the context of genomic instability and loss of cell cycle checkpoints, telomerase can fuel cancer progression.

Telomerase is expressed in nearly 90% of human tumors, making its detection extremely promising for aiding in cancer diagnosis and prognosis, as well as treatment. As the telomerase holoenzyme is composed of multiple components, there are many potential targets for achieving telomerase inhibition (Fig. 2). *In vitro* models have proven very useful for identifying the most promising telomerase inhibitors and for assessing the effects of telomerase inhibition on

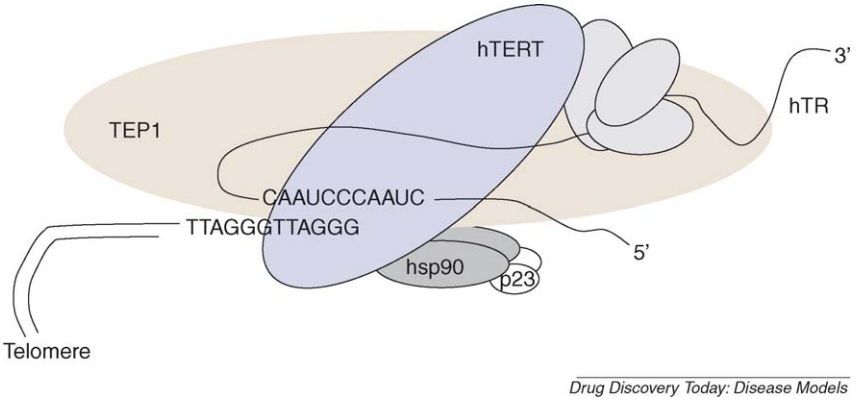

Figure 2. Schematic of telomerase, its components and how it functions at the telomere. Telomerase is a ribonucleoprotein enzyme that consists of two core components, hTR (template RNA) and hTERT (catalytic subunit). Other associated proteins include telomerase associated protein (TEP-1; no known function); hsp90 chaperone complex (p23 and hsp90; modulate telomerase assembly) and the RNA (hTR) binding proteins (hnRNPs; snoRNAs; in marble). Telomerase elongates telomeres by utilizing the 11 base hTR template to add nucleotides to the 3' overhang at the end of chromosomes.

Table 1. Comparison of *in vitro* models of telomerase inhibition

Model	Types	Target	Biological effect	Problems	Refs
Antisense	Oligonucleotides 2'-MOE oligomers[a] Peptide nucleic acids	hTR, hTERT	Senescence Apoptosis	Uptake into the cells Stability within the cells Target hybridization Lag period	[20,25]
Dominant negative protein	Multiple RT[b] motif mutants	hTERT	Senescence Apoptosis	Long lag period	[20,25]
Ribozymes	Hairpin Hammerhead	hTR, hTERT	Apoptosis	Incomplete knockdown of target	[26]
siRNA	Synthetic Short hairpin	hTR, hTERT	Apoptosis	Incomplete knockdown Target-dependent lag period	[4,20]
N3'→P5'-thio-phosphoramidate	GRN163L	hTR	Senescence	Lag period (shorter than other treatments)	[11]
Mutant template hTR	47A, AU5	hTR	Apoptosis	Low expression levels within the cell	[7]

[a] 2′-*O*-(2-Methoxyethyl) oligomers.
[b] Reverse transcriptase motif.

the growth and viability of cancer cells (Table 1). Ribozymes and short-interfering RNA (siRNA) are useful in knocking down proteins *in vitro*, but the knockdown, either at the protein or RNA level, is not 100% complete. However, the most recent studies indicate that complete knockdown is not essential for efficient and rapid apoptosis in reference to siRNA against hTR and ribozymes targeting hTERT. By contrast, the GRN163L compound and the mutant template human telomerase RNA (MT-hTR) have also displayed great effectiveness by inducing apoptosis in culture and in mouse models [7,11]. In fact GRN163L, used in combination with chemotherapy or radiotherapy, has the potential to inhibit outgrowth of residual cancer cells and treatment of micrometastatic disease [11]. It is still unclear what makes a cell undergo senescence versus apoptosis in response to telomerase inhibition. One cellular fate over another is probably owing to the ability of the cell to recognize the crucially short telomeres as damaged DNA and 'manage' this literal telomeric shortcoming. The tendency for cancer cells to undergo apoptosis might reflect frequent deficiencies in the p53 pathway, which is crucial for induction of senescence, thus triggering p53-independent apoptosis as a default mode in response to dysfunctional telomeres.

In vitro model systems have also been useful at identifying telomere binding proteins and defining their role in telomere length regulation and telomere hierarchical structuring. The telomere repeat binding factors (TRF) 1 and 2 directly bind to double strand telomeric repeats whereas POT1 (protection of telomeres) associates with the single stranded 3′ overhang [12]. More recently, additional targets have been identified that are recruited to the telomere and associated with telomere binding proteins rather than telomeric DNA. Several of these protein–protein interactions involve components of DNA repair pathways, suggesting that telomeres might serve as storage sites for DNA repair proteins. As telomeres appear particularly vulnerable to oxidative damage, this stable association with repair proteins would allow for efficient repair and/or suppression of inappropriate telomere fusion events, which could have severe biological consequences for the cell.

In the case of cancer cells, targeting telomeres for such dysfunction is a desirable strategy. Studies are being conducted with therapeutic agents being used (i.e. Adriamycin) [13] or tested (i.e. Radicicol) [14] in the clinic as well as using molecular strategies (i.e. dominant negative mutants of TRF2) [15] to better understand how telomere dysfunction is caused and what are the downstream events triggering cell death versus senescence. Certainly the continued use of isogenic cell lines established to block, stimulate or facilitate telomere capping, DNA repair pathways and cell cycle check points will be important for further elucidating the molecular mediators and consequences of telomerase inhibition.

In vivo models: the mouse

Currently the most widely utilized *in vivo* model for human disease and aging is the mouse, *Mus musculus*. In these inbred strains of mice, telomerase activity is present in all of its adult tissues with telomere lengths ranging from 40–80 kb, which is much longer in comparison with humans, 10–15 kb [16]. Unlike humans, telomere attrition is not the primary reason for cellular senescence in mice [17]. Furthermore, mouse cells are more easily transformed and immortalized, at least partially owing to the long telomeres and elevated telomerase activity [17]. In aging human populations, carcinomas (breast, lung and colon) typically cause the majority of cancer-related deaths, whereas in mice, lymphomas and sarcomas develop at the highest frequency. This species-specific difference in the tumor spectrum has been suggested to be a consequence of diversity in telomere lengths and disparity in

the regulation of telomerase. The presence of telomerase in somatic cells in mice most probably prevents the generation of dysfunctional telomeres and in turn carcinomas [16].

The most powerful model thus far, for revealing the impact of short telomeres in humans, has been the telomerase knockout mouse, which has a deletion of the murine TR (mTERC) gene that codes for the RNA component of telomerase. These mice are viable for only a finite number of generations (G6) owing to telomere loss and dysfunction [18]. There are many age-associated phenotypes such as infertility, immunosuppressive-related diseases, heart failure, diminished tissue renewal and widespread degeneration of tissues [16,19]. The inactivation of telomerase results in age- and generation-dependent telomere shortening in association with genetic instability exhibited as chromosomal fusions and elevated cancer frequency. Reconstitution of telomerase rescued many of the pathologies associated with telomere dysfunction [16].

Several mouse models have been engineered to partially recapitulate human aging. Knockout mouse models of human genetic diseases associated with premature aging and cancer predisposition normally do not replicate the human disease phenotype, such as Ataxia telangiectasia (ATM), Werner syndrome (WRN) and Bloom syndrome (BLM). Recapitulation of the human disease in mice only occurs when the mTERC gene is also deleted [19,20], thus, signifying the contribution of short telomeres and resulting in genetic instability to the pathobiology of disease. Other knockout mice that show premature aging phenotypes related to telomere length deficiencies are those eliminating the telomere binding proteins *Ku*86 autoantigen-related protein 1 (KU 86) and DNA-dependent protein kinase catalytic subunit (DNA-PKcs). In both mouse models, long telomeric fusions were observed owing to the loss of telomere capping at the ends of the chromosomes, indicating the importance of proper telomeric function and structure [21]. By contrast, human diseases associated with aging that are characterized by increased proliferation, such as cancer or atherosclerosis, are not reproduced in the telomerase deficient mouse, indicating that the development of these diseases requires further perturbations [20].

The telomerase deficient mouse has also revealed many important aspects of telomere maintenance and its significance for tissue homeostasis. This model system has provided evidence that short telomeres contribute to the inhibition of tumor progression and are more sensitive to radiation and/or genotoxic agents than long telomeres. In late generation mTERC−/− mice, p53 levels are increased, indicating that p53 senses the reduced length and signals the DNA damage response resulting in the termination of growth and/or apoptosis [17]. In addition, they are more radiosensitive in comparison with wild-type controls as seen by reduced DNA repair and by more occurrences of chromosomal damage [21]. The shortened telomeres interfere with the normal repair of double strand breaks in the genome as evidenced by the increased radiosensitivity. Consequently, perhaps the activation of the DNA damage response pathways further hinders cellular functions *in vivo* by collaborating with dysfunctional telomeres [16].

Several studies with the mTERC−/− mouse have suggested other roles for telomerase, aside from telomere elongation, based on the fact that telomerase is upregulated in mouse tumorigenesis even with the presence of extensively long telomeres [18]. Furthermore, it was discovered that, following skin chemical carcinogenesis, the telomerase deficient mouse was less prone to the development of skin tumors than wild-type mice. Therefore, regardless of telomere length in the mice, the lack of telomerase had a negative effect on tumor growth in the presence of an intact p53 DNA damage checkpoint [18,21]. From these data we can conclude that telomerase might sustain tumor growth and survival by at least two distinctive mechanisms within cancer cells. First, telomerase might signal proliferation and promote growth independently of telomere length. Second, telomerase might rescue cells with crucially short telomeres [19].

By contrast, the current mouse models for studying human cancer and aging do not always recapitulate the physiological and pathological conditions developing in humans. To develop a more accurate model system, additional studies should be conducted using a mouse model with telomeres more human-like in length. *Mus spretus* is a wild-derived or outbred species of mice with telomere lengths approximately 5-25Kb shorter than *M. musculus,* which is about the same length as humans. Another option would be to use a different vertebrate system entirely, such as chickens, which undergo senescence associated with telomere shortening in culture [22]. *In vitro*, artiodactyls (sheep and deer) and primates have also displayed telomere shortening and replicative aging [23].

However valuable the telomerase knockout mouse has been in contributing the understanding of disease and aging, there are problems with this model system. Specifically, several generations must pass before abnormalities occur, owing to the fact that the inbred strain *M. musculus* has such long telomeres. Another fundamental issue with this model system is the mTERC−/− mice do not have telomerase in any cell including those cells normally expressing telomerase activity in mice and humans (germ and stem cells). Accordingly, this complete lack of activity is discordant with the physiological status of telomerase and telomeres in humans. With respect to aging models, inconsistencies in the transition from animal to human also exist. Welle *et al.* [1] discovered that only one-third of gene expression between mice and humans displayed analogous age-related changes. Human disease phenotypes associated with premature aging and cancer predisposition are not recapitulated in mouse knockout models without additional deletion of telomerase.

Table 2. Comparison summary table

	In vitro models	In vivo models
Pros	Cost efficient, expedient Capable of high-throughput screening Use of actual human cancer cell lines Provide proof-of-principle information	Allows application of *in vitro* treatments Enables looking at effects of knockout of one or more specific genes Models multiple aspects of telomere maintenance and stability Models aspects of cancer progression
Cons	Growth conditions might affect gene expression Does not always provide complete knockdown of target Unable to model complexities of cancer within the body	Costly and time-consuming Differences in responses of gene inactivation as compared to humans Unable to develop a complete model of all aspects of cancer and aging diseases
Best use of Model	Anti-telomerase treatment development and testing	Aging models: BLM, WRN, ATM with knockout of hTERC
How to get access to the model	Literature	Literature
References	[4,7,11,20,25–26]	[1,16–23]

Therefore, alternate model systems must be developed that accurately simulate the progression of human aging and cancer.

Model comparison

In vitro models are particularly useful for providing proof-of-principle data, whether the approach involves genetically manipulating target cells to directly test a hypothesis or for high-throughput, cost-effective screening purposes (Table 2). Data generated *in vitro* often provide the fundamental basis for developing *in vivo* models, for which there is no substitute. Developing disease-specific, biologically relevant experimental systems is perhaps one of the greatest challenges for scientists, with the fields of telomeres and telomerase being no exception. Despite the limitations of inbred strains of mice, much has been learned particularly relating to the biological consequences of gradual telomere shortening at the organismal level. Generating mice that are telomerase deficient in the context of aging syndrome defects has added to our understanding of the dynamic interplay between crucially short telomeres and defects in DNA response and repair. The relative ease with which cells lines can be established from transgenic mice provides additional *in vitro* tools for dissecting molecular mechanisms.

For many years there has been a disconnect between the well-established replicative senescence of cultured cells and organismal aging, with some still arguing that the Hayflick Limit is merely a tissue culture artifact. Clearly, genetically defined inbred strains of mice have bridged a gap between *in vitro* model systems and the progressive processes of aging and cancer development in humans. Knockout animal models have also been a powerful system for confirming that short telomeres significantly affect a cell's proliferative potential and functional status. Both mouse knockout models and *in vitro* systems have validated the paradigm that telomeres can both suppress and facilitate cancer, based on the cellular context. Without a telomere maintenance mechanism, telomere dysfunction ensues fueling the genomic instability that facilitates cancer progression. However, without telomerase or an alternate telomere maintenance mechanism, cancer cells can no longer divide (i.e. short telomeres suppress tumor progression).

It has been assuring to discover that independent studies using *in vitro* and *in vivo* model systems have come to similar conclusions. Certainly with the development of more suitable *in vivo* model systems (i.e. where telomerase is more tightly regulated and telomeres are more human-like in length), there will come a more complete picture of the multiple roles of telomeres and telomerase in the context of human aging and cancer.

Models translated to humans

Experimental systems have taught us that ectopic telomerase can protect against genomic instability, a driving force in both aging and cancer. So now we must ask, 'How can this knowledge be applied to humans to prevent cancer and aging?' (see Outstanding issues). Certainly a better understanding of telomerase regulation will be necessary before seriously considering such an approach. Theoretically, if hTERT could be activated with a small molecule or ubiquitously expressed in somatic cells early in life, this could prevent age-related genomic instability and the vast majority of cancers [24]. Multiple telomerase inhibition strategies *in vitro* have successfully halted cancer cell growth. Now we must question, 'Can telomerase inhibitors be delivered selectively to cancer cells so that stem cell function is not compromised?' and 'Recognizing the lag period between inhibition and meaningful biological effects, is this approach robust enough to realistically combat a large tumor burden or is it more appropriate as a neoadjuvant therapy?' (see Outstanding issues). A more realistic approach might be using telomerase inhibition as a means to sensitize cancer cells to more standard therapeutic modalities, hence, the adjuvant treatment option. In the clinic, the focus has been on the selective expression of telomerase, using a pan-tumor-specific antigen for therapeutic vaccines; selective activation of the

hTERT promoter to trigger tumor cell killing; and administration of GRN163L for telomerase enzyme inhibition, just to name a few strategies [24].

Conclusions

Clearly, *in vitro* models lead scientists toward understanding the signaling and regulatory mechanisms involved in human disease, whereas *in vivo* systems allow us to apply the knowledge of these mechanisms to understanding the phenotypic and organismal consequences of disrupting or altering these pathways. As studies move forward, the continued practice of utilizing multiple models will be crucial. As no experimental system perfectly mimics human disease, we as scientists must capitalize on a model's strength, recognize its limitations and be hopeful that interspecies differences will lead us to discovery rather than merely frustration.

Outstanding issues

- How can the use of ectopic telomerase expression in cell culture be applied to humans to prevent cancer and aging?
- Can telomerase inhibitors be selectively delivered to cancer cells so that stem cell function is not compromised?
- Recognizing the lag period between inhibition and meaningful biological effects, is antitelomerase therapy robust enough to realistically combat a large tumor burden or is it more appropriate as a neoadjuvant therapy?

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